

Immunomodulation during sublingual therapy in allergic children

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The clinical efficacy of sublingual immunotherapy (SLIT) has been demonstrated, but its mechanism of action is still controversial. The most recent experimental observations suggest that a critical role in the modulation of immune response is sustained by Th2 cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13, by co-stimulatory molecules, such as CD40 on B cells, and by hormones and neuropeptides. To better understand whether SLIT affects immune responses we used a double-blind placebo-controlled design. Eighty-six children with mild asthma due to allergy to *Dermatophagoides pteronyssinus* (33 of whom also had rhinoconjunctivitis) were randomly assigned SLIT ($n = 47$) or placebo ($n = 39$). We assessed symptom scores using diary cards of each patient and determined the expression of CD40 on B cells and the serum concentration of ECP, IL-13, prolactin (PRL) and ACTH at enrolment and after 6 months of therapy. We observed a significant reduction in asthma and rhinitis scores in the immunotherapy group compared with the placebo group, no variation in CD40 and ACTH, but a significant decrease in ECP, IL-13 and PRL after 6 months of therapy ($p < 0.01$). Our results confirm the efficacy and safety of SLIT, and lead us to believe that it could modulate the synthesis of Th2 cytokines, as revealed from the decrease of IL-13. In addition, the reduction of PRL might be a signal of reduced activation of T lymphocytes.

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It is known that allergic reactions are sustained by Th2-cytokines as well as by co-stimulatory signals, such as CD40, thus allergic patients are characterized by an increased synthesis of cytokines, such as interleukin-4 (IL-4) and IL-13 (that induce B cells to switch towards IgE and IgG4 synthesis) and IL-5 (that enhances activation of eosinophils and release of ECP) (1–4). Recent studies show that IL-13 is important in the production of memory cells; in atopic subjects the synthesis of IgE seems to be more dependent on IL-13 than in healthy controls (5).

Specific immunotherapy has been shown to affect the Th2/Th1 ratio by means of a 'reorientation' of the immune system from a Th2-dependent reaction to allergens (6). Sublingual immunotherapy (SLIT) is nowadays considered clinically safe and effective (7–9); despite this, its mode of action is still controversial: no modulatory mechanism has been demonstrated as in

subcutaneous immunotherapy, and none of the immunologic parameters studied (IL-4, IL-5, CD40, etc.) have been the object of research during sublingual therapy, so further investigations about its mechanism of action need to be done (10).

Many findings have recently indicated that also hormones and neuropeptides, such as prolactin (PRL), ACTH, enkephalines, endorphines, and other POMC-derived peptides, play an important role in immune responses, modulating them by means of a neuroendocrine mechanism: host defence against infection, trauma and shock relies heavily on the neuroimmunoregulatory network, and the synthesis of IgE is also controlled by neuropeptides (11, 12). PRL is also produced by activated T lymphocytes: *in vitro*, it acts as an autocrine and paracrine growth factor for previously stimulated immunocompetent cells (13); but, its role *in vivo* is still the object of

investigations. ACTH and glucocorticoids down-regulate the immune response and, *in vitro*, glucocorticoids induce a decrease of specific IgE and IgG4 (14).

The aim of this study was to better understand the mechanism of action of SLIT. Thus, we evaluated several markers of allergic inflammation: ECP, IL-13; and PRL and ACTH, two hormones that influence the modulation of immune system.

Materials and methods

Patients

We used a double-blind, placebo-controlled design according to good clinical practice rules, after approval by the Ethics Committee and Italian Health Authorities.

We selected 86 children allergic only to *Dermatophagoides pteronyssinus*. The children included in the study, 35 females and 51 males, were between 5 and 12 years old (median age: 10). They all had a history of mild/moderate asthma (33 of them also had rhinoconjunctivitis), a positive skin prick test with a wheal diameter > 5 mm to house-dust mites (HDM) (*D. pteronyssinus* – Lofarma, Italy) and specific IgE to HDM at least of class 3 (RAST method, Pharmacia, Uppsala, Sweden). Only patients with a FEV_1 greater than 70% of predicted were included; methacholine inhalation tests to determine airway responsiveness were performed according to the method described by Cockcroft et al. (15) and showed no significant differences between treated and controls. Patients were excluded if they had a positive skin test to other inhalant allergens, a clinical history of other allergies, such as seasonal asthma due to pollens, a history of immunotherapy in the previous year, or severe asthma. The demographic data of the children being studied are shown in Table 1.

The children enrolled in the study were randomly assigned SLIT ($n = 47$) or placebo ($n = 39$). Before beginning treatment there was a 3-month period of observation for all patients, to assess the symptom score before therapy accord-

ing to the scale described by Tari et al. (16). FEV_1 (% of predictive) and metacholine responsiveness of all patients were measured before therapy. During the study patients were allowed to take other drugs for relief of symptoms, if needed, for no more than 7 consecutive days: inhaled steroids (200 µg/puff, 2–4 puffs) and inhaled salbutamol (250 µg/puff, 1–3 puffs) on demand. Patients were asked to record daily symptom scores in diary cards according to the same scale used before beginning immunotherapy. After 6 months of treatment all children were subjected to a clinical reevaluation, all diary cards were evaluated and FEV_1 was measured.

Clinical protocol

In our study we used the sublingual vaccine by Alk-Abellò (Milan, Italy); informed consent was obtained from the parents or guardians. The treatment protocol consisted of graded courses of a standardized house-dust extract or placebo in the same glycerine/phenol diluent followed by maintenance therapy (17). The extract was graded in five strengths: 0.016, 0.08, 0.4, 2.0 and 10 BU/ml. The Der p1 and Der p2 contents of 10 BU/ml allergenic extract were 4 and 2 µg/ml, respectively. Patients received increasing doses from each vial, beginning with one drop from the 0.016 BU/ml vial and increasing 1 drop a day for 5 days; the vaccine was taken sublingually in the morning before breakfast, kept in the mouth for at least 2 min and then swallowed. The procedure was then repeated with each following vial until the maximum dose (5 drops from vial no. 5, 10 BU/ml) was reached. The maintenance dose was 5 drops of 10 BU/ml three times a week (corresponding to 2.4 µg/ml of Der p1 and 1.2 µg/ml of Der p2 per week) until the end of the trial.

Blood samples

We collected blood samples from each patient before the beginning of the treatment and after 6 months of SLIT; all samples were collected at the same time (08.00 hours) to avoid the physiological circadian variations.

To determine CD40, we used fluorescent mAbs purchased from the Coulter Corp. (Miami, FL, USA). We separated peripheral blood mononuclear cells in conformity with Boyum's method: 10 ml of heparinated venous blood was diluted 1 : 1 with phosphate-buffered solution (PBS) and centrifuged using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) at 1600 g for 15 min. The mononuclear cells were collected, washed twice

Table 1. Demographic data of the study groups

	Therapy group n = 47	Placebo group n = 39
Median age (range)	9 (5–12)	9 (7–11)
Sex (M/F)	28/19	22/17
Rhinoconjunctivitis	18	15
Mean (SD) duration of disease (years)	1.8 (0.6)	1.6 (0.5)

with PBS and suspended in PBS at 10^5 cells/ml. Next, 400 μ l was incubated with 10 μ l of mAb anti-CD40 for 60 min at $+4^\circ\text{C}$, then washed and suspended again in PBS and analyzed by means of an EPICS XL-MCL flow cytometer (Coulter Corp.).

Concentration of ECP and IL-13 was assessed using an enzyme-linked immunosorbent assay (ELISA), while PRL and ACTH concentrations were measured using chemiluminescence and immunoradiometric assays (IRMA), respectively. Each serum was aliquoted in three samples: ECP assay was carried out on fresh sera, while the other sera were stored at -80°C . We used standardized assays purchased from Pharmacia (Uppsala, Sweden) for ECP (measuring range 2–200 ng/ml), from Euroclone Ltd. (UK) for IL-13 (sensitivity <1.5 pg/ml), from Boehringer Mannheim (Germany) for PRL (sensitivity 0.47 ng/ml) and from IDS Ltd. (UK) for ACTH (sensitivity 0.55 pg/ml). All assays were carried out according to manufacturer's instructions.

Statistical analysis

We tested our data using nonparametric tests: we used the Mann–Whitney *U*-test for comparisons between groups and Wilcoxon's test for comparisons within groups; the tests comparing two entities were two-tailed. The Student's *t*-test was used to compare the diary card data and the respiratory function results. We used a Bonferroni's correction for multiple analyses. *p*-Values less than 0.05 were considered to have statistical significance. All laboratory data are expressed as median with, between parentheses, the values of the 25th and 75th percentile, while asthma and rhinitis scores and FEV_1 (percentage of predicted) are expressed as mean values.

Results

Clinically, as can be seen in Table 2, we registered a considerable improvement of the symptoms in the treated children compared with the placebo group. Mean symptom scores for asthma and rhinitis decreased significantly; in addition, a

Table 2. Mean symptom scores and FEV_1 values before and after therapy

	Asthma scores		Rhinitis scores		FEV_1	
	T_0	T_1	T_0	T_1	T_0	T_1
SLIT*	3.28	1.28	0.84	0.39	83.4	92.6
Placebo**	3.08	3.15	0.91	0.82	80.7	81.2

p* < 0.001; *p* NS.

T_0 = mean before therapy; T_1 = mean of 6 months of therapy.

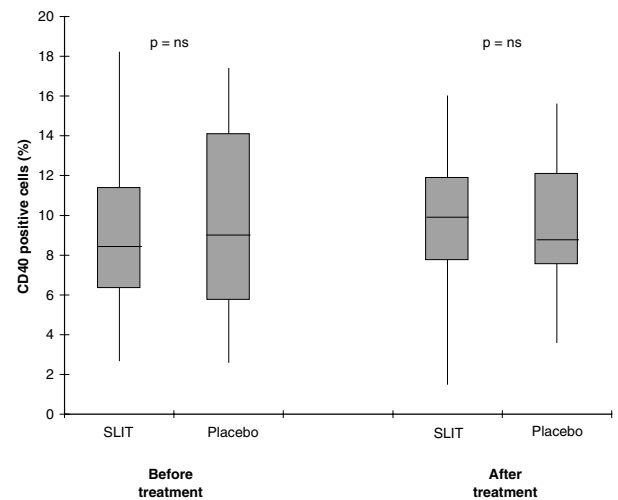


Fig. 1. Percentage of CD40+ B cells in patients and controls before and after sublingual immunotherapy (SLIT) or placebo (NV < 10%).

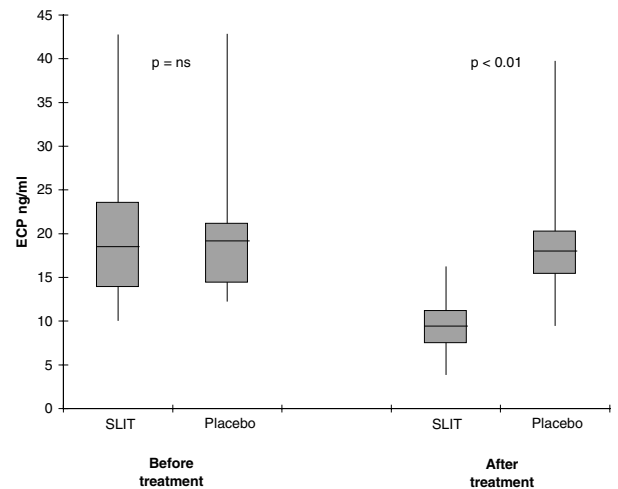


Fig. 2. Serum ECP concentration in patients and controls before and after sublingual immunotherapy (SLIT) or placebo (NV < 12 ng/ml).

significant improvement in FEV_1 was observed after 6 months of immunotherapy. The tolerance was excellent: there were no side-effects and no patient interrupted therapy.

The percentage of CD40 positive B cells was not affected by SLIT: before therapy it was 8.5% (6.3–11.4) in the treated group and increased to 10% (7.7–11.9), while in the control group it was 9.1% (5.7–14.1) before treatment and 8.7% (7.5–12.1) after 6 months of SLIT or placebo, respectively. There was not significant difference between treated and controls at both times (Fig. 1).

All treated patients showed a significant reduction in the serum concentration of ECP

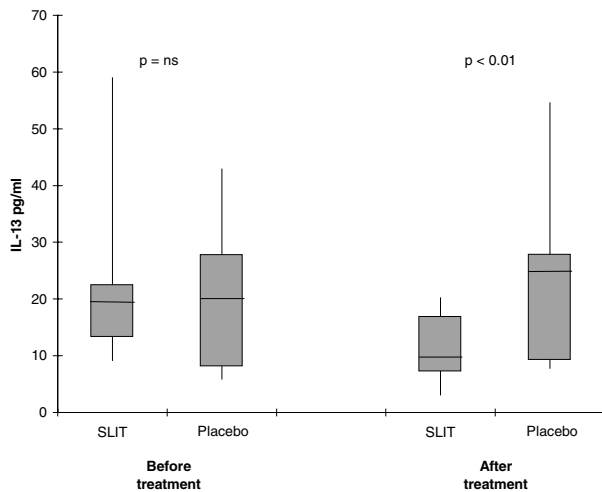


Fig. 3. Serum IL-13 concentration in patients and controls before and after sublingual immunotherapy (SLIT) or placebo (NV < 10 pg/ml).

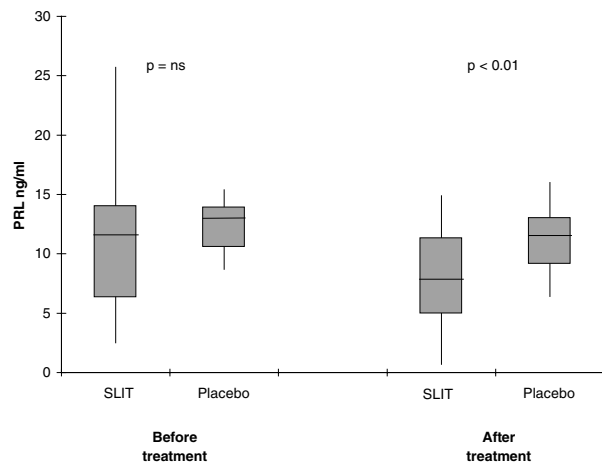


Fig. 4. PRL concentration in patients and controls before and after sublingual immunotherapy (SLIT) or placebo (NV < 15 ng/ml).

(Fig. 2). The decrease of serum ECP after 6 months of therapy was 18.5 ng/ml (13.8–23.6) before SLIT and 9.5 ng/ml (7.4–11.2) after 6 months ($p < 0.001$) in the active group, while in the placebo group the serum concentration of ECP was 17.9 ng/ml (15.3–20.3) ($p < 0.01$).

Before treatment, the concentration of IL-13 was 17.3 pg/ml (13.1–22.5) in the therapy group and 17.8 pg/ml (8.0–27.8) in the control group; after 6 months, it was 8.45 pg/ml (7.1–16.9) and 21.9 (9.1–27.9), respectively, statistically significant with intra- and inter-groups analyses (Fig. 3).

Before SLIT, the concentration of ACTH was 3.85 pg/ml (1.5–5.8) in the treated group and 4.17 pg/ml (0.73–8.93) in the control group, and its variation after 6 months was not statistically

significant. In contrast, the concentration of PRL decreased in the therapy group (Fig. 4): at baseline it was 11.65 ng/ml (6.28–14.05) in the treated patients and 12.8 ng/ml (10.5–13.95) in the controls. After 6 months, in the active group we observed a median concentration of 8.7 ng/ml (4.9–11.35) and this result was statistically significant ($p < 0.01$), while in the placebo group the concentration of PRL was 11.9 ng/ml (9.1–13.05), not statistically significant with respect to baseline, but significantly higher than in the active group ($p < 0.05$).

Discussion

The clinical efficacy and safety of SLIT has been previously demonstrated (7–10), but most of the published studies were performed on seasonal allergy or on small groups of HDM-sensitized patients. Mungan et al. compared subcutaneous and sublingual immunotherapy vs. placebo in a double-blind study in which 36 patients were enrolled and found that in the sublingual group there was a significant reduction in the rhinitis score but a nonsignificant decrease in the asthma score (18). More recently, Bahceciler and colleagues observed a significant reduction in the asthma score in children allergic to HDM treated with SLIT, but they enrolled just 15 patients (8 receiving SLIT and 7 receiving placebo), so their data need to be evaluated in a larger group (19).

In our trial we enrolled 87 children, 47 of whom received SLIT. Patients (or their parents) recorded all daily symptoms on diary cards that were evaluated after 6 months of therapy; both rhinitis and asthma scores were the mean of the recorded symptoms and significantly decreased in the SLIT group. In addition, a significant increase in FEV_1 was observed in the treated patients; this is an objective parameter that confirmed the scores from diary cards.

In addition, there were no relevant side-effects, neither local nor systemic, and the compliance of the patients and their parents was excellent: after 6 months no patient interrupted SLIT.

These findings leads us to believe that SLIT is effective and safe; both subjective (diary cards) and objective (FEV_1) parameters confirm its efficacy and sublingual therapy may be considered a valid alternative to subcutaneous immunotherapy especially in children.

Despite the excellent clinical outcome, the mechanism of action of SLIT is still unknown: our results might help to better understand the immunologic modulation as a basis for its efficacy. As described above, we observed a

decrease in ECP, IL-13 and PRL, whereas ACTH and CD40+ B cells were unchanged.

It is known that serum ECP (as well as its concentration in bronchoalveolar lavage fluid and nasal secretions) is increased in atopic subjects with respect to healthy subjects, and, among atopics, in asthmatic patients with respect to nonasthmatics, and that it is correlated with clinical score (20,21). All the treated children showed a noticeable reduction in the serum concentration of ECP; these results confirm that ECP is a valid marker of allergic inflammation (22,23).

The role of the co-stimulatory molecule CD40, expressed on B cells, in the pathogenesis of allergy is well known and confirmed by our observations in subcutaneous immunotherapy (24). Our data do not show a significant variation in the percentage of CD40 positive B cells: a possible explanation might be that in the mucosa, where the allergens penetrate during SLIT, processing and presenting of antigens preferentially involves interactions between Langerhans' APC and TCR γ/δ + T lymphocytes. Consequently, SLIT, in which allergens penetrate through the mucosa, could not influence the expression of CD40 on B cells.

The decrease of IL-13 and PRL is particularly interesting. IL-13 promotes the production of memory cells and, together with IL-4, the switch towards IgE and IgG4. The decrease of IL-13 in the treated group leads us to believe that SLIT seems effective in promoting the reorientation of the immune response by a shift in Th2/Th1 balance (2,5,6).

PRL has a stimulating function both in ontogenesis and in modulation of the immune system, even if its role *in vivo* is not yet clear (11,13,25). This hormone is also produced by T cells and plays a role in facilitating the expansion of lymphocyte clones; it is a second messenger for IL-2 and induces phosphorylation on some of the proteins of the TCR/CD3 complex (13,26,27). In addition, Hosokawa et al. showed that, *in vitro*, PRL induces an over-expression of the γ/δ TCR and Zuany-Amorim et al. observed that γ/δ + T cells are essential for inducing IL-4-dependent IgE and IgG1 responses and for Th2-mediated airway inflammation (28,29). The decrease in PRL concentration might be an expression of a reduced activity of Th2 lymphocytes, especially if we consider this result together with the decrease in IL-13. The reduction might also facilitate down regulation of airway inflammation inhibiting the expression of γ/δ TCR in the mucosa. The concentration of PRL was in the normal range even before treatment and this finding suggests

that SLIT does not influence its endocrine function and that the role of PRL has to be evaluated in the light of an excessive Th2 response.

In conclusion, we observed that SLIT was effective in reducing rhinitis and asthma symptoms due to allergy to *D. pteronyssinus*; this reduction was confirmed by increases in FEV_1 in the treated patients. In addition, we observed a decrease in ECP, IL-13 and PRL that leads us to believe that sublingual therapy might modulate the Th1/Th2 balance, inhibiting the Th2 lymphocytes, and that PRL might act locally in the mucosa, contributing to the reduction in allergic inflammation. It may be interesting to further evaluate these parameters in larger (multicenter) trials and in comparative studies with subcutaneous therapy, to better understand the different mechanisms of action of both allergen-specific immunotherapies.

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